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(54) Polypeptides exhibiting skeletal growth factor activity.

(57) Polypeptide compositions are provided which when introduced into a host enhance bone growth, as well as alkaline and acid phosphatase levels *in vivo*. The polypeptides may also be used in diagnostics for preparation of reagents or antibodies. The polypeptides range in molecular weight from about 10,000 to about 200,000dal.

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POLYPEPTIDES EXHIBITING SKELETAL GROWTH FACTOR ACTIVITY

5   For several years, scientists have suspected that there was localized regulation of resorption of bone and growth of bone cells. See Farley et al., Program and Abstracts of 61st Annual Meeting of the Endocrine Society (1979); Howard and Baylink, Clinical
10 Research (1980) 28:50A; and Howard et al., Calcif. Tissue Int. (Suppl.) (1980) 31:53 as illustrative. The possibility of there being a localized factor which controls bone growth and resorption offers the opportunity to isolate a material which could be
15 effective in the therapeutic control of bone growth in a wide variety of situations. Such a growth factor might be useful in the treatment of osteoporosis, in conjunction with bone implants, for the production of reagents and antibodies in diagnostic assays, and in
20 the investigation of bone growth, bone replacement and bone deterioration.

        It is therefore of great importance to be able to provide compounds capable of being used for diagnosis and therapy of bone in the mammalian host.
25 Description of the Prior Art

        Farley and Baylink, Biochemistry (1982) 21:3502-3507; and Farley et al., ibid. (1982) 21:3508-3513 describe skeletal growth factor from human bone, its isolation and characterization. See also,
30 Baylink and Farley, Abstract of Paper given at the XVII European Symposium on Calcified Tissues, April 11-14, 1983 describing the isolation and partial purification of a putative coupling factor from human bone. Other abstracts which are cumulative of the above disclosures
35 include Howard et al., Calcif. Tissue Int. (Suppl.) (1982) 34:S30; Murphy et al., ibid. (1982) 34:S26; Farley et al., ibid. (1982) 34:S38; and Wergedal and

Baylink, _ibid_. (1982) _34_:516. Urist _et_ _al_., _Clin_.
Orthopaedics _and_ _Rel_. _Res_. (1982) _162_:219-231 and
Sampath _et_ _al_.: Role of extracellular matrix in local
bone induction. In: Current Advances in
5 Skeletogenesis. Eds. Silbermann and Slavkin,
Amsterdam, Exerpta medica (1982) describe small factors
which differ in properties or activities from the
subject peptides.

SUMMARY OF THE INVENTION

10 Novel polypeptides ranging over a wide
molecular weight range are provided having
physiological activity _in_ _vitro_ and _in_ _vivo_ as skeletal
growth factors. The polypeptides are found to enhance
bone cell growth, while also enhancing levels of bone
15 and serum alkaline and serum acid phosphatase. The
compounds can be used to promote the proliferation of
bone cells, _in_ _vitro_ and _in_ _vivo_, potentially in the
treatment of osteoporosis, in treatment to increase
longitudinal bone growth in children with short
20 statute, and for the preparation of reagents for use in
diagnosis of the level of skeletal growth factors in
serum or other tissue.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Novel polypeptides of varying molecular
25 weights are provided which when administered to bone
cells induce a number of physiological effects. Bone
cell proliferation occurs, which _in_ _vivo_ enhances bone
formation and matrix apposition rate. Osteoclastic
resorption is increased, as well as an increase in
30 forming surface. Serum levels of alkaline phosphatase
and tartrate insensitive acid phosphatase are enhanced,
which increases are consistent with an increase in
osteoblast number and bone formation. The polypeptides
are active with vertebrates, such as mammals and birds,
35 quadrapeds and bipeds, e.g. humans, rats, chickens,
etc., as well as with fish. There appears, therefore,

to be a similar mechanism in bone growth regulation over a wide spectrum of species.

The polypeptides include naturally occurring polypeptides derived from native bone, analogs of such
5 polypeptides demonstrating one or more of the physiological characteristics of the native polypeptides and fragments thereof.

The subject polypeptides may be divided into three molecular weight categories: (1) about
10 100-200kdal (kilodaltons), usually 150-200kdal; (2) about 80-85kdal; and (3) about 10-20kdal.

The "high" molecular weight (150-200kdal) or large skeletal growth factor ("SGF") may be obtained from bone, particularly portions of hip bone, such as
15 femoral heads or other portions of the hip. The bone parts are cleaned, pulverized in liquid nitrogen, demineralized and extracted with a chelating agent, e.g. ethylenediaminetetraacetic acid (EDTA), conveniently in combination with a biostatic or
20 biocidal agent. Particularly, from about 5% to 25% EDTA with about 0.01 to 0.1% sodium azide may be employed, more particularly from about 10% to 20% EDTA and from about 0.03 to 0.05% sodium azide. Alternatively, the bone can be demineralized with HCl
25 (0.1 to 1.0M) and extracted with aqueous solution. The extracts may then be concentrated and desalted, at least in part. Purification procedures then involve one or more of the following steps: Chromatography, e.g. on Sephadex or DEAE Sephacel; gel filtration, e.g.
30 on Agarose, particularly Agarose 0.5M; and HPLC ion exchange chromatography.

The large SGF may be characterized by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions, where it migrates as a
35 single major band with bovine serum albumin tetramer, while providing multiple bands in the 50 to 90kdal range on SDS PAGE. Also, multiple bands are observed

on isoelectric focusing in the acidic range, pH 4 to 6. At about physiologic pH (pH 7.2) the high molecular weight SGF binds to hydroxyapatite, QAE cellulose, and DE-52, but not CM-cellulose. The large SGF is stable

5 to denaturing agents, such as dithiothreitol, urea and guanidine-HCl, while losing its activity upon hydrolysis with trypsin.

The "middle" molecular weight or medium SGF may be prepared in part as described above, except

10 differing in that after treatment with EDTA, the dispersion is heated for a short period at an elevated temperature, generally in the range of about 75 to 85°C for about 15 to 30min, cooled, and insoluble protein separated and discarded. The solution is then

15 subjected to acid precipitation (pH ~3), and the acid soluble protein neutralized to substantially neutral pH, concentrated and chromatographed (gel filtered) on Sephadex, e.g. Sephadex G-200.

The procedure may be found in greater detail

20 in Farley and Baylink (1982), _supra_.

The middle molecular weight SGF gives a single band that migrates just behind bovine serum albumin monomer on PAGE under non-denaturing conditions, multiple bands on SDS PAGE in the 50-70kdal

25 range and an isoelectric point of 6.5. The middle molecular weight SGF is stable to collagenase, β-mercaptoethanol and butanol, but loses it activity upon treatment with trypsin or urea.

The "low" molecular weight or small SGF is

30 obtained in substantially the same manner as the large SGF. The amount of the small SGF may be enhanced by preincubation prior to HPLC gel filtration and is found to be unbound or weakly bound to DEAE Sephacel and binds to hydroxyapatite. This small SGF fraction is

35 not observed in the preparation of human SGF, but is found in the preparation of bovine and chicken SGF. The compositions obtained from bovine and chicken are

believed to contain a protease which results in the
fragmentation of the large SGF to provide for the small
SGF fractions. Treatment of human SGF with the
protease found in other species or modifying the

5   isolation procedure may provide for the small SGF
observed with the other species.

The subject compositions can be used in a
wide variety of ways, both for diagnosis and therapy.
For diagnosis, the compounds may be used as antigens

10   for the production of antibodies specific for the
particular types of growth factor. The antibodies may
then find application both in diagnosis and therapy.
Alternatively, the polypeptides may be used as reagents
in diagnostic assays, where the polypeptides may be

15   labeled or unlabeled. Similarly, for use in diagnosis,
the antibodies may be labeled or unlabeled. A wide
variety of assays can be employed using one or both,
the antigen or antibody, either labeled or unlabeled.
Various labels include fluorescers, enzymes,

20   chemiluminescers, radionuclides, substrates, cofactors,
or the like. Assays in which the reagents may be
employed include hemagglutination, radioimmunoassay,
enzyme immunoassay, both homogeneous and heterogeneous,
fluorescence immunoassays, substrate-linked

25   immunofluorescence assays, or the like. These assays
may be found in numerous U.S. patents, such as U.S.
Patent Nos. 3,817,837; 4,133,639; 4,134,792; 4,160,016;
and 4,172,117. The relevant disclosures of these
patents are incorporated herein by reference as to the

30   preparation and use of the various reagents.

The subject compounds may also be used for
the study of bone-related cells in the determination of
available receptors, responsive cells to the presence
of the SGF, or the like. Toward this end, the subject

35   compounds can be used in vitro for enhancing the
proliferation of bone cells responsive to the SGF.

In situations where there is a deficiency of SGF in the serum or the patient is suffering from osteoporosis or other bone disease or when implanting bone, where it is desirable to enhance the bone

5   proliferation in a particular area, the subject compounds can be locally administered or administered intraperitoneally, intravenously or subcutaneously. Administration of the SGF or antibodies tc SGF may be in any convenient formulation in accordance with the

10   purpose of such administration and the manner of administration. Where the protein administered will be generally distributed in the blood system, the dosage will generally be in the range of about 0.005 to about 0.5mg/g of host, depending upon the efficiency of the

15   SGF, duration in the host and the potential for localization of the SGF in the host. The SGF may be administered neat or as a solution of from about 0.01 to 10 weight percent in a physiologically acceptable carrier, e.g. water, PBS-saline, or the like.

20   The subject compounds can also be used for determining receptor populations of bone cells and to modulate the growth rate of bone cells in vitro.

The following examples are offered by way of illustration and not by way of limitation.

25   EXPERIMENTAL

The preparation of human skeletal growth factor was performed as follows. Human femoral heads, (obtained at hip replacement surgery) were frozen in liquid nitrogen, crushed with a Wiley mill and

30   repeatedly rinsed with 25mM phosphate buffer, pH 7.2, to remove all contaminating serum and marrow. The washed bone fragments were then suspended in a small volume of 10% EDTA, 0.04% sodium azide, at pH 7.2 (about 10mL/100g original weight of bone) and dialysed

35   against the same solution at 4°C. In order to obtain the high molecular weight hSGF, the retentate is desalted with Sephadex G-25, chromatographed on DE-52

(DEAE Sephacel) and then subjected to Biogel A (0.5M)
gel filtration after which time the material is treated
to HPLC ion exchange chromatography followed by HPLC
TSK 3000 gel filtration.

5 The resulting product which is found to have
a molecular weight in a range from about 150,000 to
200,000dal migrates as a single major band on PAGE
under non-denaturing conditions, migrating with bovine
serum albumin tetramer, while multiple bands in the
10 50kdal to 90kdal range are observed on SDS PAGE and on
isoelectric focusing in the acidic range (pH 4 to 6).
At pH 7.2, the high molecular weight hSGF binds to
hydroxyapatite, QAE cellulose and DEAE Sephacel, but
not carboxymethyl cellulose. The large hSGF is stable
15 to denaturing agents such as dithiothreitol, urea and
guanidine HCl, while activity is lost upon treatment
with trypsin.

  The 83kdal hSGF is obtained by somewhat
different conditions as described in Farley and Baylink
20 (1982), supra.

  Bovine skeletal growth factor (bSGF) can be
prepared from the hip bones from freshly slaughtered
cows. The bones are mechanically scraped clean of soft
tissue and cut into 2cm³ sections. The bone sections
25 are frozen in liquid nitrogen and ground in a Wiley
mill to yield a bone powder that is washed with warm
water to remove fat and serum protein, demineralized
and extracted using 20% EDTA, 0.04% sodium azide as
described above. The EDTA extracts are concentrated
30 and partially desalted by Amicon ultrafiltration. The
remaining EDTA is removed by desalting with Sephadex
G-25. Three alternative methods can be used for
further purification of the desalted crude extracts.
(1) Gel filtration on Agarose yields an active bSGF
35 fraction of 150 to 200kdal. (2) Direct purification of
the crude extract on DEAE Sephacel yields a large bSGF
fraction that is modestly bound. In addition, active

bSGF fractions that are unbound or weakly bound to DEAE
Sephacel are obtained in the range of 10 to 20kdal.

(3) Purification of desalted crude extract on
hydroxyapatite yields a non-active fraction that elutes

5   with 0.15M phosphate in an active fraction that is
recovered by dissolving the hydroxyapatite support with
EDTA. The tightly bound fraction yields approximately
equal amounts of large bSGF and small bSGF.

The large partially purified bSGF tends to

10 become small on further manipulation. Evidence
suggests that an endogenous bone protease is present in
the bone extracts and is activated during the
purification process. This protease is evident in the
bovine bone extracts and as will be indicated

15 subsequently in chicken extracts, but is not observed
in the purification of the human extracts.

Chicken skeletal growth factor (cSGF) is
prepared from the tibia and femur of adult chickens.
The bones are mechanically scraped clean of soft

20 tissue, the cartilage ends are cut off, the bones are
frozen in dry ice and smashed into 3mm^3 pieces. Serum
proteins and fat are removed by extensive washing of
the bone pieces in 0.03M tris(acetate), 0.15M NaCl, pH
7.4, with vigorous agitation. The bone is then

25 subjected to demineralization and extraction with 10%
EDTA, 0.04% sodium azide. The EDTA extracts are
concentrated and partially desalted by Amicon
ultrafiltration and the remaining EDTA removed by
Sephadex G-25 chromatography. Two approaches are used

30 to further purify the cSGF. (1) Gel filtration of the
desalted crude extract on Agarose 0.05M yields
predominately a large form of cSGF (100 to 200kdal), as
well as small quantities of the small cSGF in the 10 to
20kdal range. (2) DE-52 chromatography of the desalted

35 crude bone extract yields a cSGF fraction that is
weakly bound to the matrix. The weakly bound fraction
behaves like large cSGF when chromatographed on a TSK

3000 HPLC gel filtration column shortly after isolation. Preincubation of the weakly bound fraction before HPLC gel filtration results in a shift of large cSGF to the smaller cSGF. This change in apparent size is believed to be a result of the presence of a protease which is detected in the fraction weakly bound to DE-52. cSGF activity in the desalted crude extracts binds to hydroxyapatite, QAE cellulose and DE-52, but does not bind to collagen linked to Agarose or to carboxymethyl Sepharose.

Pig, salmon, and rat bones can also be processed as described above for the chicken bones. The desalted crude extracts from these three species causes a substantial increase in cellular proliferation when tested on bone cells from chick calvaria.

The extracts were then employed in a number of tests to demonstrate their activity as compared to other known growth factors as well as to determine their intrinsic activity.

The first study was a comparison of the SGF growth factors with a number of other growth factors. (See Table 1.)

TABLE 1: Comparison of SGF with other known growth factors

Property	GROWTH FACTOR				
	SGF	EGF	FGF	IGF-1	PDGF
M.W.	150-200K[a]	74K[c]	13.5K	60-150[c]	35-38K
	83K[b]	6K		7-9K	
IEP	4-5	5.4	6 / 8-9.5	8-8.6	9.8
Stability:					
Heat	+	+	-	+	-
Acid	+	+	-	+	
Reducing Agents	+	-	+	-	-
Trypsin	-		-		-
Target Cells	Bone	Ectoderm Endoderm	Mesoderm 3T3	Mesoderm Fibroblast 3T3, Bone	Smooth Muscle
Source	Bone	Submaxillary Gland	Pituitary Brain	Liver, Serum CSF	Serum Platelet

SGF: Skeletal Growth Factor 1[a], 2[b]   EGF: Epidermal Growth Factor[3]
FGF: Fibroblast Growth Factor[4],[5]   IGF: Insulin-like Growth Factor[6],[7]
PDGF: Platelet-derived Growth Factor[8]

IEP: Isoelectric Point   c: Exist as complex with binding proteins

TABLE 1 (continued): Comparison of SGF with other known growth factors

Property	ECGF	MGF	NGF	TGF
		GROWTH FACTOR		
M.W.	75K	45-54K	140K	30-60K
	17-25K	18-23K	26-44K	10K
IEP	4-6	Acidic		
Stability:				
Heat	+	+		+
Acid	-		-	+
Reducing Agents				-
Trypsin		+		-
Target Cells	Endothelial 3T3	Lung Fibro-	Nerve	
Source	Hypothalamus	Lung	Salivary Submaxil-lary Gland	Transformed Cells

FDGF: Endothelial Cell Growth Factor[9] MGF: Macrophage Growth Factor[10,11]
NGF: Nerve Growth Factor[12] TGF: Transforming Growth Factor[13,14]
IEP: Isoelectric Point

12

References for Table 1

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The above Table 1 compares a number of physical properties of the various growth factors showing similarities and differences between the various growth factors.

5   In the next study, the SGF was tested in combination with a number of other growth factors or mitogens on 3T3 cells using ^{3}H-thymidine incorporation as a measure of cell proliferation. The following Table 2 indicates the results.

10

Table 2: Effects of Combination of hSGF with Other Polypeptide Growth Factors

	% of hSGF Activity		
Factor	−hSGF	+hSGF	Significance
Control	9.4 ± 2.4	100.0 ± 19	p<0.001
EGF (10ng/ml)	13.7 ± 3.0	92.0 ± 27	p<0.001
FGF (10ng/ml)	70.9 ± 13.2	136.6 ± 46.6	p<0.01
IGF (25ng/ml)	30.2 ± 4.6	84.3 ± 13.7	p<0.001
PDGF(20ng/ml)	34.8 ± 2.3	75.3 ± 9.9	p<0.001

25 3T3 cells were plated at 70,000 cells/well in DMEM in 24 well culture dishes. 24 hours after plating, the factors were added and incubated for an additional 24 hours. The cells were then pulse labeled for 2 hours with 2µCi of ^{3}H-TdR and TCA precipitable radioactivity was quantitated. Incorporation is expressed as % of maximum incorporation stimulated by hSGF alone and is the average of 6 repli-
30 cates.

Comparisons are made between the maximum stimulation of the same cells with hSGF alone to the
35 maximum stimulation by the four mitogens in the presence and absence of hSGF. hSGF alone stimulates cell proliferation seven-fold greater than EGF,

three-fold greater than IGF-I and PDGF, and 40% greater
than FGF. In addition, the maximum stimulation of the
four mitogens was significantly elevated by the
addition of hSGF. These results indicate that hSGF is
5 non-competitive and different from the four mitogens
tested.

In a similar experiment, stimulaticn of chick
embryonic calvarial cell proliferation by fetal calf
serum at different serum concentrations (0.05%, 0.1%,
10 0.5%, 1.0%, and 2.0%) was significantly elevated by the
addition of hSGF. The maximum stimulatory effect of
serum (1050% ± 133% of control with 1.0% added serum)
increased by 27% (p<0.001) in the presence of hSGF.

The above results suggest that hSGF is
15 distinct from the other known growth factors that were
tested and from the growth promoting agents in the
serum (polypeptide growth factors as well as
fibronectin and transferrin).

Experiments were carried out in vivo with
20 rats. Rats were given intraperitoneally daily
injections of bovine bone extract containing 2.5mg of
protein for a total of ten days. Biochemical and
quantitative histological comparisons were made between
the control rats and rats treated with bovine bone
25 extract. The results of this experiment are summarized
in Table 3.

Table 3

Experiment 1: A comparison of several parameters in control rats versus rats treated with bovine bone extract.

Parameter	Control	Treated[a]	p[b]
Serum Calcium (mg%)	9.8(±0.7)	9.0(±0.5)	NS[c]
Body Weight (Start)	104(±3.7)	105(±4.2)	NS
Body Weight (End)	147(±4.9)	147(±9.2)	NS
Femur Alkaline Phosphatase (mU/mg protein)	4.9(±0.9)	11.6(±1.2)	<0.001
Femur Acid Phosphatase Tartrate Insensitive (mU/mg protein)	3.4(±0.4)	4.8(±0.3)	<0.02

a   All data reported as mean ±S.E.M. and based on observations from 5 rats in the control group and 3 rats in the treated group.

b   Comparisons of treated versus control were by the students T test.

c   Not significant at the p<0.05 student T test.

Table 3 (continued)

Experiment 1: Quantitative histological comparisons of femur and vertebral sections from control rats and rats treated with bovine bone extract.

Parameter	Control	Treated[a]	p[b]
Tibiae Periosteal Bone Formation (mm^3)	0.23(±0.02)	0.40(±0.02)	<0.001
Tibiae Periosteal Bone	6.75(±0.43)	8.60(±0.46)	<0.05
Tibiae Medullary Cavity (mm^3)	0.67(±0.03)	0.81(±0.09)	<0.05
Vertebral Forming Surface (single + double label) % total surface	58.3 (±2.5)	90.5 (±1.7)	<0.001
Vertebral Neutral Surface (no label) % total surface	41.7 (±1.9)	9.5 (±1.4)	<0.001

a All data reported as mean ±S.E.M. and based on observations from 5 rats in the control group and 3 rats in the treated group.

b Comparisons of treated versus control were by the students T test.

c Not significant at the $p < 0.05$ student T test.

This experiment was performed with five rats in the control group and three rats in the treated group. Serum calcium and final body weight were not altered by the treatment. The femur alkaline

5 phosphatase level, a probable index of bone formation, and the tartrate insensitive acid phosphatase, a possible index of osteoclastic cell number, were both increased in the treated rats. Quantitative histological comparisons of the femur clarified the

10 results of the enzymatic analysis. Tibia periosteal bone formation as well as the matrix apposition rate were clearly increased in the treated rats. Tibia medullary area, an index of osteoclastic resorption, was also increased in the treated rats. In addition,

15 histological analysis of the vertebral sections gave a highly significant increase in forming surface of the treated rats.

The next study was a more detailed study on the effects of bovine bone extract in vivo. In this

20 study, ten rats were used in both the control and treated groups. (See Table 4.)

Table 4

Experiment 2: A comparison of several parameters in control rats versus rats treated with bovine bone extract.

Parameter	Control[a]	Treated[a]	p[b]
Serum Calcium (mg%)	10.7(±0.3)	11.1(±0.3)	NS[c]
Serum Phosphate (mg/dl)	4.7(±0.5)	5.8(±0.2)	<0.05
Serum Alkaline Phosphatase (mU/mg protein)	63.7(±2.2)	91.7(±5.2)	<0.001
Body Weight (Start)	86.4(±0.2)	85.0(±0.6)	NS
Body Weight (End)	149.8(±2.6)	147.7(±2.5)	NS

a All data reported as mean ±S.E.M. and based on observations from at least 10 rats in each group.

b Comparisons of treated versus control were by the students T test.

c Not significant at p<0.05.

Table 4 (continued)

Experiment 2: Skeletal alkaline phosphatase and tartrate insensitive acid phosphatase levels in the femur, skull and sternum from control rats and rats treated with bovine bone extract.

	Alkaline Phosphatase[a] (mU/gm bone wet weight)			Acid Phosphatase[a] Tartrate Insensitive (mU/gm bone wet weight)		
	Control	Treated	p	Control	Treated	p
Femur	155(±24)	284(±19)	<0.001	31(±5)	60(±7)	<0.001
Skull	405(±44)	1075(±84)	<0.001	355(±42)	607(±52)	<0.001
Sternum	46(±2)	93(±7)	<0.001	107(±16)	137(±14)	NS

a All data reported as mean ±S.E.M. and based on observations from at least 10 rats in each group.

b Comparisons of treated versus control were by the students T test.

c Not significant at p<0.05.

Serum calcium and body weight did not change with treatment. However, both serum alkaline phosphatase (p<0.001) and serum phosphate (p<0.05) were significantly increased in the treated rats. The
5   levels of alkaline phosphatase in the femur, skull and sternum of treated rats were elevated (p<0.001). Tartrate insensitive acid phosphatase was also increased in the femur. and skull (p<0.001), but not the sternum. The results of the alkaline phosphatase
10   analysis in the femur, skull and sternum are consistent with an increase in osteoblast number and bone formation. The alkaline phosphatase data agrees very well with the quantitative histology of the tibiae of treated rats, where a 60% increase (p<0.001) in the
15   periosteal bone formation (mm³) and a 40% increase in the matrix apposition rate (µM/day) were observed.

Table 5:   Experiment 2 - Quantitative Histological Comparisons of Tibiae Sections from Control Rats and Rats Treated with Bovine Bone Extract

Parameter	Control	Treated[a]	p[b]
Tibiae Periosteal Bone Formation (mm²)	0.45(±0.02)	0.72(±0.04)	<0.001
Tibiae Periosteal Bone Apposition Rate (µM/day)	8.58(±0.33)	12.07(±0.51)	<0.001

25   a   All data reported as mean ± S.E.M. and based on observations from at least 10 rats in each group.

b   Comparisons of treated versus control were by the students T test.

The increase in tartrate sensitive acid
30   phosphatase in the femur and skull is consistent with an increase in bone resorption.

A low molecular weight SGF (approximately 11 kdal) was isolated and purified from both human and bovine bones, as follows. Human femoral heads obtained during hip replacement surgery and bovine bone (pelvis

5 and femur) obtained from a local abattoir were cleaned to remove the adhering soft tissue, cut into small pieces with a band saw, washed with water to remove marrow and fat, ground in a Wiley mill, washed with deionized water containing protease inhibitors to

10 remove blood, and then treated with 4M guanidine hydrochloride (pH 7.4) with protease inhibitors to extract the soluble cellular constituents, serum proteins and soluble collagen. The resulting human bone residues were then demineralized, and the

15 noncollagenous proteins extracted 4-5 times with 10% EDTA containing 4M guanidine hydrochloride and protease inhibitors (EDTA-guanidine).

Purification of the 11 kdal SGF from the human bone EDTA guanidine extracts was as follows. The

20 extracts were subjected to hydroxylapatite chromatography which, in the presence of 4M guanidine, will pass the SGF while binding other noncollagenous proteins. The unbound fraction from the hydroxylapatite chromatography was then subjected to

25 high pressure liquid chromatography (HPLC) on a preparative gel filtration column. Using 4M guanidine hydrochloride in 30mM Tris acetate (pH 7.4) as an eluent, it was found that the 11 kdal SGF eluted after myoglobin (17.5 kdal) and before aproteinin (6.2 kdal).

30 The HPLC fraction containing the 11 kdal SGF was then desalted and purified by HPLC reverse phase chromatography using a silica-based C_4-bonded phase column which separates proteins based on their hydrophobicity. Proteins bound to the silica were eluted by increasing

35 the concentration of acetonitrile in the eluent

(water:trifluoroacetic acid:acetonitrile). The above
extraction and purification procedures are specifically
for the human skeletal growth factor. Purification of
the bovine skeletal growth factor involved only minor
5 modifications of the procedure used for the human
factor.

The purity of the 11 kdal human SGF was
measured at each stage of purification, and the results
are set forth in Table 6 below.

10 Table 6: Human Skeletal Growth Factor Purification Table[*]

Step	Protein (Mg)	Specific Activity (Units/μg Protein)	Activity Recovered (%)
Guanidine EDTA	322	14	100
Hydroxylapatite (Fast Flow)	168	25.5	95
HPLC Gel Filtration	43.5	75.0	72
HPLC Reverse Phase I	1.86	945	39
HPLC Reverse Phase II	0.21	4000	18.6

[*] 360 g of human femoral head bones were used as starting
material. Activity of SGF was assayed by its effect on bone cell
20 proliferation. SGF stimulates the incorporation of ^{3}H-Thymidine
(Tdr) into TCA precipitable material in embryonic chick calvaria
cells. 100% increase in ^{3}H-Tdr incorporation over unstimulated
control was defined as one activity unit.

The homogeneity of both the human and bovine
25 11 kdal SGF fractions was demonstrated by the following
observations. The SGF was shown to elute as a single
symmetric peak upon HPLC reverse phase separation. The
SGF was shown to migrate as a single band upon
polyacrylamide-sodium deodecyl sulfate (SDS)-urea gel

electrophoresis which effects separation based on
molecular weight. The SGF was also shown to migrate as
a single band upon electrophoresis using a 5-15%
polyacrylamide gradient gel where separation is
5 effected based on differences in molecular size.
Finally, the SGF was shown to migrate as a single band
upon isoelectric focusing on a 7.5% polyacrylamide gel
where separation depends on differences in the isoelec-
tric points. Thus, the material in the 11 kdal SGF
10 fraction was shown to have uniform molecular weight,
molecular size, and isoelectric point.

The molecular weight of the purified SGF
species was determined to be 11±1 kdal by polyacryl-
amide-SDS-urea gel electrophoresis and HPLC gel fil-
15 tration using 4M guanidine hydrochloride (pH 7.4) as an
eluent. The large molecular weight (83 kdal) species
of SGF can be dissociated into the 11 kdal species and
a carrier protein in the presence of 4M guanidine
hydrochloride. The isoelectric point of the 11 kdal
20 SGF was determined to be 6.8±0.2, and was shown to be
sensitive to reducing agents such as dithiothreitol.
The 11 kdal SGF was shown to be sensitive to trypsin,
but resistant to treatment with collagenase and with
acid (0.1% trifluoroacetic acid, pH 2.5), 6M urea, and
25 4M guanidine hydrochloride.

The 11 kdal SGF was shown to be a very potent
mitogen for bone cells with a half-maximum activity
reached at 2 to 3 ng/ml. The mitogenic activity was
assayed by observing the incorporation of ^{3}H-Tdr into
30 TCA precipitable material (DNA) in embryonic chick
calvarial (bone) cells. hSFG at 0.1 ng/ml, the
activity (expressed as a percentage incorporation of
[3]H-Tdr to that of untreated control) was approximately
150%. The activity reached a maximum of approximately
35 400% at between 5 and 10 ng/ml. The maximum activity
did not further increase as the concentration was
increased to 100 ng/ml. bSFG showed similar

activities. Preliminary studies of the amino acid
composition of bovine SGF indicate that the 11 kdal SGF
is rich in aspartic acid, glutamic acid, leucine, and
proline. The amino acid analysis is set forth in Table
5 7 below.

Table 7:   Amino Acid Analysis of Bovine SGF (Residues/Molecule)

	Lysine	6
	Histidine	3
	Arginine	3
10	Aspartic Acid	9
	Threonine	3
	Serine	5
	Glutamic Acid	9
	Proline	6
15	Glycine	5
	Alanine	4
	Valine	5
	Isoleucine	4
	Leucine	7
20	Tyrosine	2
	Phenylalanine	3
	Total No. of Amino Acid Residues	75

Comparisons of the molecular weight, chemical
characteristics, and amino acid composition of the 11 kdal
25 SGF with other known polypeptide growth factors indicate
that the 11 kdal SGF is a unique mitogen different from
other known growth factors.

The 11 kdal SGF has also been demonstrated to
enhance bone formation in vivo in each of four separate
30 experiments with ten rats per group in each experiment.
Eleven kdal SGF which had been purified by EDTA extraction
and hydroxylapatite chromatography from a bovine bone
extract was administered to groups of rats by daily intra-
peritoneal injection for a period of 10 days. Control rats
35 received BSA injections, and (in order to assess the rate of
bone formation) all animals were injected with tetracycline
on the first and tenth days. Tetracycline is a highly
fluorescent substance that tightly binds to bone as it

mineralizes. Thus, only skeletal surfaces that are undergoing bone formation become fluorescent. Animals were sacrificed after the tenth day, and one tibia from each rat was histologically evaluated for bone formation, including

5 measurements of surface fluorescence. A portion of the other tibia was extracted with a butanol solution to release alkaline phosphatase from the plasma membranes of the osteoblasts. Alkaline phosphatase is regarded as a specific marker for the osteoblasts which are bone forming cells.

10   The amount of tetracycline labelled bone surface was increased in the tibiae of rats that received daily injections of the 11 kdal SGF compared to the BSA controls. The percentage of increase varied from a low of approximately 140% of control for one experiment to a high of

15 approximately 240% of control for another experiment. The rate of bone formation on the external (periosteal) surface of the tibia was shown to be greater in rats injected with the 11 kdal SGF than in controls. The skeletal alkaline phosphatase activity in the bone samples taken from rats

20 treated with 11 kdal SGF were also shown to be increased from a low approximately 140% in one experiment to a high of approximately 210% in another experiment. Taken together, these results clearly demonstrate that the 11 kdal SGF is a potent mitogen capable of systemically enhancing bone

25 formation in mammals.

It is evident from the above results that the novel skeletal growth factors provided by the subject invention can be used for a multitude of purposes in enhancing the proliferation of bone cells, both _in vivo_

5   and _in vitro_, diagnosing for the presence of these skeletal growth factors in serum or other sites, and in the treatment of various bone diseases, where there is a need for stimulation of bone growth.

Although the foregoing invention has been

10   described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced.

CLAIMS:

1. A vertebrate skeletal growth factor of from about 100,000 to 200,000dal or fragment thereof, capable of stimulating a proliferation of bone cells.

5

2. A skeletal growth factor according to Claim 1, wherein said vertebrate is mammal, and said growth factor is of from about 150,000 to 200,000dal.

10

3. A skeletal growth factor according to Claim 1, wherein said mammal is human and said skeletal growth factor is of from about 150,000 to 200,000dal.

4. A skeletal growth factor according to

15 Claim 1, wherein said skeletal growth factor is a fragment of from about 10,000 to 20,000dal.

5. A skeletal growth factor according to Claim 1, wherein said vertebrate is human.

20

6. A skeletal growth factor according to Claim 1, wherein said vertebrate is bovine.

7. A skeletal growth factor according to

25 Claim 1, wherein said vertebrate is chicken.

8. A method for enhancing the proliferation of bone, said method comprising:
contacting bone cells with an effective

30 amount of a skeletal growth factor according to Claim 1.

9. A method according to Claim 8, wherein said vertebrate is human.

35

10. Antibodies capable of binding to a vertebrate skeletal growth factor according to Claim 1.

11.   Antibodies according to Claim 10, conjugated with a label capable of providing a detectable signal.

5   12.   A method for determining the presence of a vertebrate skeletal growth factor according to Claim 1, which comprises:

    combining a sample suspected of containing said skeletal growth factor with an antibody according
10 to Claim 10, and determining the occurrence of binding of said antibody to said skeletal growth factor.